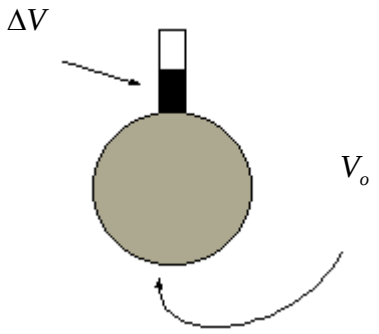
Appendix: Equations Used for Fitting ITC Data

I. General Considerations

It will be assumed throughout that the macromolecule M is in the cell at an initial bulk concentration M_t^o (moles/liter) before the first injection, and the ligand X to be injected is initially at zero concentration in the cell. The *working volume* (cross-hatched area below) of the lollipop-shaped cell is V_o, the size of the i^{th} injection is ΔV_i and the total liquid which has been injected at any point during the experiment, ΔV, is simply the sum of the individual ΔV_i for all injections.



At the beginning of an experiment, both the cell and the long communication tube are filled with macromolecule solution, but it is only that contained within V_o that is sensed calorimetricallly, Because of the total-fill nature of the cell each injection acts to drive liquid out of the working volume and up into the inactive tube as shown by the darkened portion representing ΔV. Thus, the concentration of macromolecule in V changes a small amount with each injection since the total number of moles of macromolecule initially in V (i.e. M_t^o times V_o) at the beginning of the experiment is later distributed in a larger volume, $V_o + \Delta V$. Since the average bulk concentration of macromolecule in ΔV is the mean of the beginning concentration M_t^o and the present concentration M_t in the active volume, then conservation of mass requires that

$$M_t^o V_o = M_t V_o + \frac{1}{2}\left(M_t + M_t^o\right)\Delta V \qquad (1)$$

so that

$$M_t = M_t^o \left(\frac{1 - \frac{\Delta V}{2V_o}}{1 + \frac{\Delta V}{2V_o}} \right) \qquad (2)$$

Using similar reasoning, it is easily shown that the actual bulk concentration of ligand in V_o, X_t, is related to the *hypothetical* bulk concentration X_t^o (assuming that all of the injected ligand remained in V_o) as follows:

$$X_t^o V_o = X_t V_o + \frac{1}{2} X_t \Delta V \qquad (3)$$

$$X_t = X_t^o \left(\frac{1}{1 + \frac{\Delta V}{2V_o}} \right) \qquad (4)$$

The above expressions for M_t and X_t are used by Origin to correct for displaced volume effects which occur with each injection.

II. Single Set of Identical Sites

In the following equations,

K = Binding constant;
n = # of sites;
V_o = active cell volume;
M_t and [M] are bulk and free concentration of macromolecule in V_o;
X_t and [X] are bulk and free concentration of ligand, and
Θ = fraction of sites occupied by ligand X.

$$K = \frac{\Theta}{(1-\Theta)[X]} \qquad (5)$$

$$X_t = [X] + n\Theta M_t \qquad (6)$$

Combining equations (5) and (6) above gives

$$\Theta^2 - \Theta\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right] + \frac{X_t}{nM_t} = 0 \qquad (7)$$

The total heat content Q of the solution contained in V_o (determined relative to zero for the unliganded species) at fractional saturation Θ is

$$Q = n\Theta M_t \Delta H V_o \qquad (8)$$

where ΔH is the molar heat of ligand binding. Solving the quadratic equation (7) for Θ and then substituting this into eq. (8) gives

$$Q = \frac{nM_t \Delta H V_o}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}}\right] \qquad (9)$$

The value of Q above can be calculated (for any designated values of n, K, and ΔH) at the end of the i^{th} injection and designated Q(i). The parameter of interest for comparison with experiment, however, is the *change* in heat content from the completion of the i-1 injection to completion of the i injection. The expression for Q in eq. (9) only applies to the liquid contained in volume V_o. Therefore, after completing an injection, it is obvious that a correction must be made for displaced volume (i.e., ΔV_i = injection volume) since some of the liquid in V_o after the i-1 injection will no longer be in V_o after the i^{th} injection, even though it will contribute to the heat effect (assuming the kinetics of reaction and mixing are fast) *before* it passes out of the working volume V_o. The liquid in the displaced volume contributes about 50%

as much heat effect as an equivalent volume remaining in V_o.[1] The correct expression then for heat released, $\Delta Q(i)$, from the i^{th} injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_o}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \qquad (10)$$

The process of fitting experimental data then involves 1) initial guesses (which most often can be made accurately enough by Origin) of n, K, and ΔH; 2) calculation of $\Delta Q(i)$ for each injection and comparison of these values with the measured heat for the corresponding experimental injection; 3) improvement in the initial values of n, K, and ΔH by standard Marquardt methods; and 4) iteration of the above procedure until no further significant improvement in fit occurs with continued iteration.

III. Two Sets of Independent Sites

Using the same definition of symbols as above for set 1 and set 2, we have

$$K_1 = \frac{\Theta_1}{(1-\Theta_1)[X]} \qquad K_2 = \frac{\Theta_2}{(1-\Theta_2)[X]} \qquad (11)$$

$$X_t = [X] + M_t(n_1\Theta_1 + n_2\Theta_2) \qquad (12)$$

Solving equation (11) for Θ_1 and Θ_2 and then substituting into equation (12) gives

$$X_t = [X] + \frac{n_1 M_t [X] K_1}{1 + [X]K_1} + \frac{n_2 M_t [X] K_2}{1 + [X]K_2} \qquad (13)$$

Clearing equation (13) of fractions and collecting like terms leads to a cubic equation of the form

$$[X]^3 + p[X]^2 + q[X] + r = 0 \qquad (14)$$

where

$$p = \frac{1}{K_1} + \frac{1}{K_2} + (n_1 + n_2)M_t - X_t$$

$$q = \left(\frac{n_1}{K_2} + \frac{n_2}{K_1}\right)M_t - \left(\frac{1}{K_1} + \frac{1}{K_2}\right)X_t + \frac{1}{K_1 K_2}$$

$$r = \frac{-X_t}{K_1 K_2} \qquad (15)$$

[1] The first infinitesimal volume element in the i injection contributes no heat effect since it has already equilibrated at existing concentrations after the i-1 injection. The last volume element of an injection contributes heat effects equal to the liquid remaining in V_o since its concentrations are equivalent to those in V_o after the i injection. Assuming linearity over the small ΔV_i volume increment, then the liquid in the displaced volume is only half as effective in producing heat relative to the liquid in V_o).

Equations 14 and 15 can be solved for [X] either in closed form or (as done in Origin) numerically by using Newton's Method if parameters n_1, n_2, K_1, and K_2 are assigned. Both Θ_1 and Θ_2 may then be obtained from equation 11 above.

As discussed earlier in section II, the heat content after any injection i is equal to

$$Q = M_t V_o \left(n_1 \Theta_1 \Delta H_1 + n_2 \Theta_2 \Delta H_2 \right) \qquad (16)$$

After a similar correction for displaced volume, the pertinent calculated heat effect for the i injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_o}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i-1) \qquad (17)$$

which may be used in the Marquardt algorithm to obtain best values for the six fitting parameters.

IV. Sequential Binding Sites

For sequential binding, the binding constants K_1, K_2, ...K_n must be defined relative to the progress of saturation, so that

$$K_1 = \frac{[MX]}{[M][X]} \quad K_2 = \frac{[MX_2]}{[MX][X]} \qquad K_3 = \frac{[MX_3]}{[MX_2][X]} \qquad (18)$$

In the sequential model, there is no distinction as to *which* sites are saturated, but only as to the total number of sites that are saturated. If the sites are identical, then there is a statistical degeneracy associated with the sequential saturation since the first ligand to bind has more empty sites of the same kind to choose from than does the second ligand, etc. For identical interacting sites then, we can distinguish between the phenomenological binding constants K_i (defined by eq (18)) and the intrinsic binding constants K_i^o where the effect of degeneracies has been removed. The relationship between the two binding constants is given by:

$$K_i = \frac{n - i + 1}{i} K_i^o \qquad (19)$$

All calculations given below, as well as parameters reported from curve-fitting, are in terms of K_i values but the operator may convert to K_i^o values, if desired, using eq (19). Since concentrations of all liganded species $[ML_i]$ can be easily expressed in terms of the concentration of the non-liganded species, [M], then the fraction of total macromolecule having i bound ligands, F_i, is simply

$$F_o = \frac{1}{P}$$

$$F_1 = \frac{K_1[X]}{P}$$

$$F_2 = \frac{K_1 K_2 [X]^2}{P} \qquad (20)$$

$$F_n = \frac{K_1 K_2 \ldots . K_n [X]^n}{P}$$

where

$$P = 1 + K_1[X] + K_1 K_2 [X]^2 + \ldots . + K_1 K_2 \ldots . K_n [X]^n \qquad (21)$$

$$X_t = [X] + M_t \sum_{i=1}^{n} i F_i$$

Once n and values of fitting parameters K_1 through K_n are assigned, then equations (20) - (21) may be solved for [X] by numerical methods (the Bisection method is used). After [X] is known, all F_i may be calculated from equation (20) and the heat content after the i^{th} injection is determined from

$$Q = M_t V_o \left(F_1 \Delta H_1 + F_2 [\Delta H_1 + \Delta H_2] + \ldots . + F_n [\Delta H_1 + \Delta H_2 + \Delta H_3 + \ldots .. + \Delta H_n] \right) \qquad (22)$$

And, as before,

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_o} \left[\frac{Q(i) + Q(i-1)}{2} \right] - Q(i-1) \qquad (23)$$

Which then leads into the Marquardt minimization routine.

V. Enzyme/substrate/inhibitor Assay

Assaying enzymes, inhibitors or substrates by calorimetric activity has the major advantage that it works well for any enzyme/substrate/inhibitor system with no prior chemical modification of any participants in the reaction. The rate R_t of the substrate decomposition reaction is directly proportional to the power output in the calorimeter cell, i.e.,

$$R_t = \frac{P}{\Delta H V_0} \tag{24}$$

where P is the power generated by the reaction, ΔH is the heat of decomposition of the substrate, and V^o is the cell volume. The units of R_t will be moles/l/sec if P is expressed in μcal/sec, ΔH in μcal per mole of substrate, and V^o in liters, for example.

If Michaelis-Menten kinetics are assumed then the experimental values for the rate R_t can be expressed as

$$R_t = \frac{k_{cat}[E]_{cat}[S]_t}{[S]_t + K_M\left(1 + \frac{[I]}{K_I}\right)} \tag{25}$$

where k_{cat} is the catalytic rate constant for substrate decomposition, K_M is the Michaelis constant, $[E]_{tot}$ is the total enzyme concentration, and $[S]_t$ is the instantaneous concentration of substrate. The equation as written is valid both in the absence or presence of a competitive inhibitor at concentration [I] and with inhibition constant K_I.

The use of equation (25) assumes no effects from product inhibition. This assumption has been discussed by Todd and Gomez (Todd, M. J. & Gomez, J. (2001) *Analytical Biochemistry* **296**, 179-187.) and found to be quantitative in many cases. In those cases where product inhibition is significant, then equation (25) can only be used to express initial rates of reaction prior to accumulation of product.

Todd and Gomez discussed in some detail the two methods by which assays can be carried out in a titration calorimeter, and these are summarized below.

Method 1: Single injection. Using this approach, the reaction is initiated by injecting enzyme solution from the syringe into the sample cell containing substrate solution, or *vice versa*. If desired, a competitive inhibitor may also be included in one solution or the other. The reaction is allowed to go to completion in the calorimeter cell, and the power P is recorded as a function of time t.

Integration of the excess power P associated with the reaction enables ΔH to be determined, i.e.,

$$\Delta H = \frac{\int_0^\infty P dt}{[S]_{t=0} V^0} \tag{26}$$

where $[S]_{t=0}$ is the starting substrate concentration. Knowing ΔH, the substrate concentration can be determined as a function of time from the equation:

$$[S]_t = [S]_{t=o} - \frac{\int_0^t P dt}{\Delta H V^0} \qquad (27)$$

After obtaining the time-dependent rate from equation (24), then these data can be equated to the Michaelis expression in equation (25) to provide the final equation to be fit by non-linear least squares. In the absence of inhibitor, k_{cat} and K_M are used as variable parameters during iterative fitting. In the presence of inhibitor I, it is best to enter previously determined values of k_{cat} and K_M and use K_I as the only variable fitting parameter.

Method 2: Multiple injections. In this method, multiple injections of substrate solution from the syringe are made into the reaction cell containing enzyme solution (with or without inhibitor). After each injection, a sufficient time is allowed for the instrument to equilibrate at the new power level resulting from the increased substrate concentration. Measurements are carried out quickly enough however so that little hydrolysis of substrate takes place relative to the total substrate contained in the cell. That is, $[S]_t$ is calculated directly from the total added substrate assuming no significant hydrolysis.

Equations (24)-(25) are still valid for Method 2, except that R_t and $[S]_t$ now correspond to *discrete* values of the rate and substrate concentration after each injection, rather than time-dependent values. To determine ΔH from equation (26), it is necessary to carry out another single-injection experiment where hydrolysis is allowed to go to completion. Having done this, then discrete values of R_t at different $[S]_t$ are calculated, so that equation (25) can then be fit to obtain best values of k_{cat} and K_M (in the absence of inhibitor). In the presence of a competitive inhibitor, data are also fit to equation (25) but using k_{cat} and K_M as fixed (results obtained from previous experiment with no inhibitor present) and treating K_I as the only fitting parameter.

VI. Dimer Dissociation Model

A protein molecule P may associate at high concentrations to form a dimer. The dilution of this concentrated protein solution by injection into the calorimeter cell containing buffer can then result in some heat effects from dissociation

$$P_2 \overset{\Delta H_{disc}}{\Longleftrightarrow} 2P \qquad\qquad K = \frac{(P)^2}{(P_2)}$$

where (P) and (P_2) are the concentrations of monomer and dimer and where ΔH_{disc} is the heat of dissociation of the dimer. It is assumed in this model that the stoichiometry is well-defined, i.e., no aggregates with stoichiometry higher than 2 are present. By measuring heats for a series of injections it is then possible, using curve-fitting, to determine the dissociation constant K, and heat of dissociation.

The equivalent monomer concentration after the i^{th} injection, C_i, is the sum of the actual monomer concentration $(P)_i$ plus 2 times the aggregate concentration $(P_2)_i$. Using the expression for the dimer dissociation constant to obtain $(P)_i$ in terms of $(P_2)_i$ leads to the equation

$$C(i) = K^{1/2}(P_2)_i^{1/2} + 2(P_2)_i \qquad (28)$$

A similar expression applies to the solution in the syringe of fixed concentration C_{syr}

$$C_{syr} = K^{1/2}(P_2)_{syr}^{1/2} + 2(P_2)_{syr} \qquad (29)$$

Since C_{syr} is known and C_i can be determined from C_{syr} knowing injection volumes, then $(P_2)_{syr}$ and $(P_2)_i$ can be determined from equations (28)-(29) if K is assigned.

The heat released q_i when the i^{th} injection of volume dV_i is made into a fixed-volume (V_o) cell will be

$$q_i = \Delta H_{disc}(P_2)_{syr}\, dV_i - \Delta H_{disc}\left[(P_2)_i - (P_2)_{i-1}\right]\left[V_0 + \frac{dV_i}{2}\right] \qquad (30)$$

The first term in equation (30) is the heat content of the aggregate contained in the injection volume prior to injection while the second term is the net heat content due to the *difference* in aggregate present in the cell before and after the injection. The $\left[V_0 + \frac{dV_i}{2}\right]$ factor in the final term is an *effective* volume which takes into account the displacement which occurs in a total-fill cell (see Appendix, section I).

Assuming experimental parameters V_o, dV_i , and C_{syr} are known, equations (28)-(30) are simultaneous equations which can be solved for q_i whenever values are assigned to K and ΔH_{disc}. Only the latter two parameters are varied during iterative fitting.

VII. Competitive Binding Model

Using conventional ITC methods, binding constants from $10^3\ M^{-1}$ to $10^8\ M^{-1}$ can be measured most accurately. When binding constants significantly exceed $10^8\ M^{-1}$, instrument sensitivity becomes challenged as concentrations are lowered to the point where quantitative measurements of the binding constant would be possible. On the other hand, binding constants substantially in excess of $10^8\ M^{-1}$ can be measured quantitatively if such strong-binding ligands are studied in competition with a second ligand which binds competitively but more weakly to the same binding site.

Competitive binding studies are carried out using the strong-binding ligand A as the injectant, with the solution in the cell containing the second competitive ligand B as well as the binding protein P (or other target molecule). This system then has two equilibria which are displaced with each injection, i.e.,

$$A + P \overset{\Delta H_A}{\Leftrightarrow} PA \qquad \mathrm{K_A} = \frac{[PA]}{[P][A]}$$

$$B + P \overset{\Delta H_B}{\Leftrightarrow} PB \qquad \mathrm{K_B} = \frac{[PB]}{[P][B]}$$

The value of K_B and ΔH_B for the competing ligand are first measured in a conventional ITC experiment, and these parameter values are entered as known parameters when determining K_A from results of the competition experiment. For the competition experiment, the total concentration of competing ligand, $[B]_{tot}$, should be selected such that

$$\frac{"K_A"}{K_B[B]_{tot}} \cong 10^5 - 10^8 \ \mathrm{M^{-1}}$$

where "K_A" is the estimated value of K_A.

The detailed equations used in the fitting model for competitive binding are found in a paper by Sigurskjold (Sigurskjold, B. W. (2000) *Analytical Biochemistry* **277**, 260-266.). MicroCal would like to thank Dr. Sigurskjold for providing this fitting routine.

VIII. Single Injection Method

Creating New Worksheet. The raw data (after time constant correction, Fourier filtering, baseline subtraction, and eliminating inappropriate data) are used to form a new worksheet which is modeled after the existing worksheet used with multi-injection binding data.

1) Input Parameters. In addition to the raw data parameters (ΔP (ucal/sec) from the Y axis and time t (sec) from the X axis of the corrected raw data, the known parameters are the injection rate R (μl/sec, stored in header), total delivery volume V_{inj} (ul, stored in header), active cell volume V_{cell} (ml), the initial macromole concentration in the cell M^o (mM) before any dilution, the dilution factor d_M for the macromolecule solution resulting from autosampler loading, the initial ligand concentration in the syringe X^o before any dilution, the dilution factor d_X for the ligand concentration resulting from loading. The approximate values are 0.95 for d_M and 0.91 for d_X, and the values will be independent of the instrument which is used in the experiment.
2) Point numbering. In the existing Origin worksheet for multiple injections, the rows are numbered 1,2,3, …. according to the injection number. In the worksheet for single injection experiments, the numbering corresponds to the data point number. The data points will be spaced at one for each filter period (2 sec).

3) DH and time t columns. The DH column corresponds to the column of the same name in the existing Origin ITC worksheet while the time t column is one which doesn't exist in the existing worksheet and must be added. The DH and time t columns should be filled with the data points from the above data set (after TC correction, FT smoothing, control subtraction, and data trimming). DH is the Y axis value ΔP (ucal/sec) and time t (sec) is the corresponding X axis value.
4) INJV column. All entries into this column should be identical and equal to the injection rate R (ul/sec) times the filter time (2 sec).
5) X_t column. $$X_t = \left(\frac{X^o d_X Rt}{1000V_{cell}}\right)\left(1 - \frac{Rt}{2000V_{cell}}\right)$$
6) M_t column. $$M_t = M^0 d_M \left(\frac{1 - \frac{rt}{2000V_{cell}}}{1 + \frac{rt}{2000V_{cell}}}\right)$$
7) XM_t column. $$XM_t = \frac{X_t}{M_t}$$

Note: Indexing for X_t, M_t, and INJV refer to values before the ith injection, while DH, XM_t, NDH refer to indexing after the ith injection (the new column time t is also indexed after the ith injection).